

USE OF ZYGOTIC EMBRYO CULTURE TO COLLECT GERMPLASM OF COCONUT (COCOS NUCIFERA L.)

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SUMMARY

The in vitro culture of zygotic embryos solves the problems encountered in transporting and storing coconut seeds (Cocos nucifera L.), especially when obtained under field collecting conditions. Embryo sampling and short-term storage methods have been developed. Direct inoculation into culture on media, enabling embryo growth under axenic conditions, has been successfully carried out under field collecting conditions. An alternative method holds embryos in endosperm cores in a salt solution for brief periods before excision and inoculation into culture in a laboratory. The techniques and equipment used are described in this paper, as well as the subsequent development of the embryos and the transfer of plants to natural growing conditions.

INTRODUCTION

Collecting and exchanging coconut germplasm are difficult and costly because of the very considerable weight and size of coconuts, their often rapid germination, and phytosanitary problems. In vitro culture of excised embryos would simplify transport and provide secure storage conditions during collection operations. In view of this, IBPGR initiated appropriate research.

Principles and examples of in vitro collecting techniques for various types of germplasm have been reviewed earlier (Withers, 1987). This paper describes the collecting technique in detail and includes improved culture data. The technique provides an interesting contrast and complement to the in vitro field explanting technique described by Sossou et al. (1987). It is simpler in concept and execution and would be more appropriate under field conditions that limit the amount of equipment and materials that could be transported.

Much research has been done on in vitro culture of coconut embryos, with the first results reported by Cutter and Wilson (1954), Abrahams and Thomas (1962) and Ventura et al. (1966). The in vitro culture of macapuno nuts (whose endosperm is semi-liquid and whose germination cannot occur under natural conditions) was then studied in detail by De Guzman's group (De Guzman, 1970; Balaga and

De Guzman, 1971; Del Rosario and De Guzman, 1976, 1981).

Different local dwarf and tall varieties have also been used for research purposes, and the development of embryos into young seedlings has been reported several times (Fisher and Tsai, 1978; Iyer, 1981; Gupta et al., 1984; Karunaratne et al., 1985). We have described the production of young seedlings from the Malayan Yellow Dwarf x West African Tall hybrid and their transfer to natural growing conditions (Assy Bah, 1986). In vitro culture methods developed on this type of material have successfully been applied to 10 dwarf varieties and 10 tall varieties (Assy Bah, 1986).

This article gives the results of studies undertaken to develop in vitro-based embryo sampling, storage and transport methods for use during collecting operations. The development of embryos in culture conditions and their transfer to natural growing conditions are also described.

MATERIALS AND METHODS

Plant material

Seeds from the Malayan Yellow Dwarf x West African Tall hybrid (PB 121 created by Centre de Coopération Internationale en Recherche Agronomique pour le Développement/Institut de Recherches des Huiles et Oléagineux), 10-12 months after germination, were used.

Equipment

1 bowl, 1 sponge, 1 small camping gas burner, 1 hammer, 1 knife, 2 pairs of forceps (30 cm), 2 cork borers (diameter 20 mm), 1 scalpel,

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soap, 1 litre of chlorine bleach, 3 or 4 500-ml bottles, a 2-litre beaker, 1 funnel, filter paper, a 1-litre flask.

For 100 nuts, the following items are required, in addition to the equipment listed above:

Equipment and consumable materials	Storage in KCl soln	Direct inoc. into culture
225 g of calcium hypochlorite	+	+
10-15 litres of water	+	+
1 rack of 100 30-ml jars each containing 15 ml sterile distilled water	-	+
20 sterile Petri dishes	-	+
2 racks of 50 25 x 150-mm culture tubes with 20 ml of medium	-	+
Plastic film	-	+
1 rack of 100 30-ml jars each containing 15 ml sterile distilled water + 16.2 g/l of KCl	+	-

Sampling and disinfection of solid endosperm cylinders containing the embryo

The first sampling step in the plantation or in the field during a collecting mission consists of isolating and disinfecting the solid endosperm cylinder containing the embryo. This operation was carried out in the open air on a table which had been carefully washed and disinfected with chlorine bleach. 2 cork borers (diameter 20 mm) and 2 pairs of forceps (30 cm) were immersed in a bowl containing a 3% chlorogenic sodium hypochlorite solution. A portable gas burner was used to sterilize the instruments.

Completely dehusked coconuts were split in two using a hammer. The half of the nut bearing the embryo was placed on the table and a solid endosperm cylinder containing the embryo removed with a cork borer (Fig. 1). The solid endosperm surrounding the embryo protects it during storage and disinfection operations; it is not removed until just before the *in vitro* culture procedure begins.

Batches of 30 cylinders were immersed for 20 min in 500-ml bottles containing a calcium hypochlorite solution (70% active chlorine; 45



Fig. 1. Removal of a solid endosperm cylinder containing the embryo

g/l) (Fig. 2). During soaking, the contents of these bottles were stirred once or twice using a pair of forceps.

Embryo storage and transport from the collecting site to the *in vitro* laboratory

Two alternative methods for handling the embryos after collection were studied: storage of the solid endosperm cylinders for subsequent



Fig. 2. Disinfection of solid endosperm cylinders



Fig. 3. Transfer of solid endosperm cylinders to a KCl solution for storage

inoculation, and direct inoculation of embryos into culture in the field under axenic conditions.

Storage of endosperm cylinders

Preliminary trials of various storage conditions led us to opt for storage in a KCl solution (16.2 g/l) for a maximum period of 14 days. Immediately after disinfection, the cylinders were transferred without rinsing to 30-ml jars containing 15 ml of the preservation solution. The forceps used to handle the cylinders were sterilized in the flame of the gas burner and the flasks were handled with their necks close to the flame to minimize introduction of microorganisms (Fig. 3). The cylinders containing the embryos are then ready to be transported to the *in vitro* culture laboratory, where the embryos will be isolated and cultured.

After the storage period, the cylinders were resterilized by transferring them individually to a filtered solution of calcium hypochlorite (70% active chlorine; 45 g/l) for 20 min. The embryos were then isolated and rinsed once in sterile distilled water before inoculation into culture on a semi-solid medium (Assy Bah, 1986).

Direct inoculation into culture in the field

All manipulations were protected by a packing case resting on its side, near a gas burner flame (Fig. 4). The forceps were heated in the flame and then cooled in the sodium hypochlorite solution in which the endosperm cylinders were immersed. The embryos were isolated in a sterile Petri dish, rinsed once in sterile distilled water (15 ml in a 30-ml jar),



Fig. 4. Direct inoculation of embryos into culture under the shelter of a packing case

then inoculated into culture on semi-solid medium prepared and sterilized beforehand.

The caps of the culture tubes were held in place by plastic film. Once inoculation has been completed, the tubes are placed in the packing case and can be transported and stored easily.

Culture conditions

The culture medium was made up from Murashige and Skoog mineral elements, iron EDTA (4 mg/l), Morel and Wetmore vitamins, sodium ascorbate (100 mg/l), sucrose (20 g/l), agar (Labosi A1540; 8 g/l) and activated charcoal (Sigma C5385; 2 g/l). The pH was adjusted to 5.5 and the medium was dispensed in aliquots of 20 ml in 24 x 150-mm culture tubes before sterilization by autoclaving at 115°C for 20 min.

Four replicates of 100 nuts per treatment were carried out. In the case of direct inoculation into culture, the embryos of 2 replicates were kept under a simple shelter for 2 months, those of the other two were placed in air-conditioned rooms at 27±1°C shortly after inoculation. They were kept in the dark until the gemmule appeared, then brought into the light (3,000 lux; 12-h photoperiod) and transferred to 35 x 300-mm culture tubes (Assy Bah, 1986).

Observations and assessment of results

Observations concentrated on the contamination rate after 1 month of culture and the percentage of embryos with a gemmule after 2 and 6 months of culture.

The results of experimental treatments were analyzed and compared with controls

Table 1. Contamination rate observed after 1 month of culturing

Replicate No.	Culturing in the laboratory (control)		Storage in KCl solution & inoculation in the lab.		Inoculation into culture at the collecting site	
	No. of embryos cultured	Contamination(%)	No. of embryos cultured	Contamination(%)	No. of embryos cultured	Contamination(%)
1	96	3.1	97	3.1	98	11.2
2	99	8.1	89	4.5	99	10.1
3	97	6.2	91	4.4	99	11.1
4	89	4.5	90	8.9	94	10.6
TOTAL	381	5.5	367	5.2	390	10.8

inoculated and cultured under laboratory conditions following the method previously described (Assy Bah, 1986). All culture treatment were compared with the usual germination of seeds in the seed bed.

The results were subjected to analysis of variance and a Duncan test after angular transformation of percentages.

RESULTS

Contamination rate

Contamination rates are given in Table 1. A highly significant treatment effect is seen (F significant at 1%). Direct inoculation into culture at the collecting site leads to a significantly higher contamination rate than the storage of solid endosperm cylinders. Nonetheless, the former rate remains relatively low at around 10%.

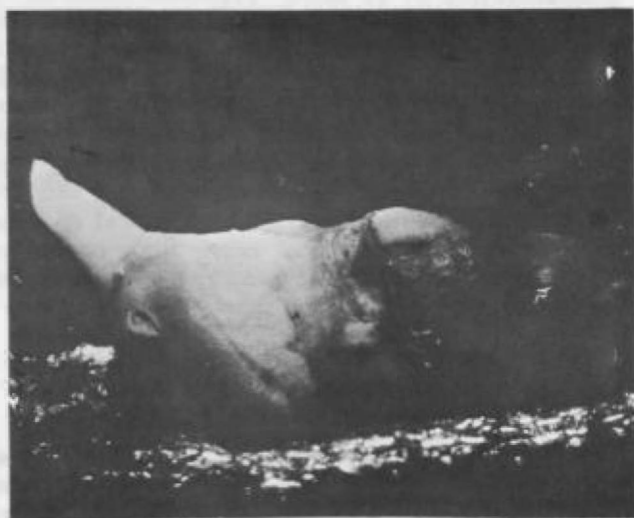


Fig. 5. Emergence of the gamule

The low rates obtained after storage of solid endosperm cylinders may be due to the fact that further disinfection is carried out just before the embryos are isolated, and to the cleaner inoculation conditions in the laboratory.

Development of excised embryos

Elongation of the embryo is observed from the first weeks of culturing. After a phase during which elongation and weight increase

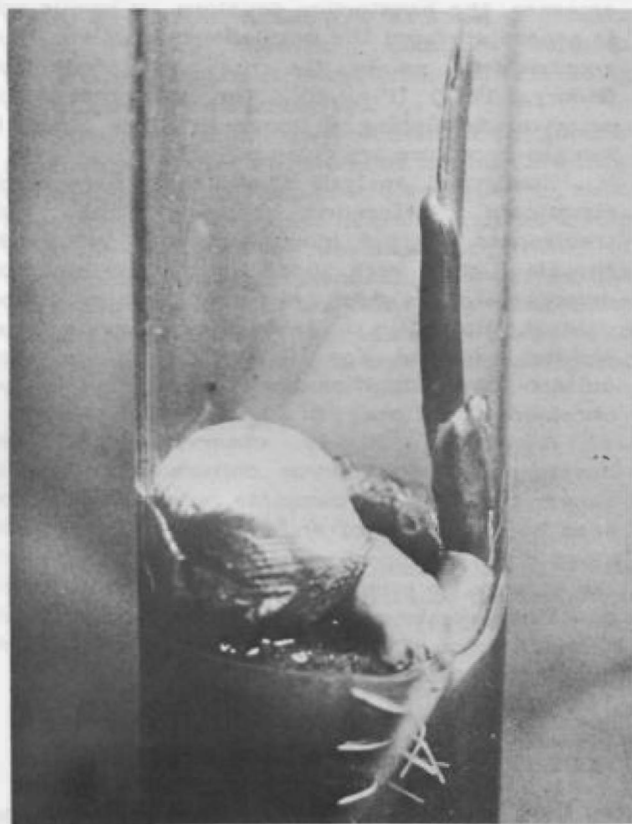


Fig. 6. Embryo development after 3 months in culture

Table 2. Percentage of embryos with a gemmule after 2 and 6 months in culture

Replicate No.	Time spent in culture or seedbed (months)	Seedbed (control)	Cultured in laboratory (control)		Storage in KCl soln and inoculation in the lab.		Inoculation into culture at the collecting site	
		Germinated nuts (%)	Uncontaminated embryos	Embryos with developed gemmule (%)	Uncontaminated embryos	Embryos with developed gemmule (%)	Uncontaminated embryos	Embryos with developed gemmule (%)
1	2	36	93	6.4	94	5.3	88	9.1
	6	97	90	63.3	90	28.9	88	71.6
2	2	30	91	8.8	85	1.2	89	2.2
	6	96	85	54.1	80	16.2	77	44.1
3	2	40	91	20.9	87	1.1	88	1.1
	6	88	90	34.4	84	28.6	80	45.0
4	2	32	85	3.5	82	0.0	84	17.9
	6	86	82	43.9	74	23.0	84	42.9
TOTAL	2	34.5	360	10.0	348	2.0	349	7.4
	6	91.7	347	49.0	328	24.4	329	51.4

occurs, the gemmule appears at the end of 2 months in culture (Fig. 5). After the gemmule emerges, the haustorium develops. It swells and is separated from the cotyledonary petiole by a constriction, as in the nut on germination (Henry, 1957) (Fig. 6). The percentages of embryos developing a gemmule after 2 and 6 months in culture are given in Table 2.

Statistical analysis shows that there is no significant difference between the two treatments. Direct inoculation into culture in the field gives very good results; the embryos demonstrate the same behaviour as those of the control cultured in the laboratory. However, the results obtained for direct inoculation into culture are significantly superior to those obtained after storage in a KCl solution.

Generally, it is observed that the development of embryos cultured *in vitro* is slower than that of complete nuts placed in the seed bed. Storage of embryos in a KCl solution slows down development even further. However, the storage, in a simple shelter, of culture tubes containing embryos inoculated into culture at the collection site (replicates 3 and 4) does not disturb gemmule development.

Transfer to natural growing conditions

Transfer to soil is the final indispensable stage in the use of *in vitro* culture for sampling, storing and transporting coconut seeds. Trials were carried out to investigate this stage.

After 4-6 months in culture, the aerial part of the plant is well developed and, in a certain number of cases, a primary root capable of giving secondary roots is observed. However, this root system, which develops spontaneously, is not sufficient to guarantee the plant's survival in soil.

Trials were conducted on the effect of different rhizogenesis-induction media. The best treatment was the basic medium previously described, with a higher level of sucrose (60 g/l) and containing naphthaleneacetic acid at a concentration of 20 mg/l. Plantlets cultured on this medium had a well-developed root system. They were transferred directly to sand under a hot-bed frame and watered for a fortnight (Fig. 7). They were then transferred to leaf mould. Initial results indicate a 79% survival rate after 15 days. Experiments have shown that the haustorium needs to be kept above the sand to ensure the success of transfer to natural growing conditions. Nonetheless, only 30% could be transferred to the nursery and their development is slow under present conditions.

DISCUSSION AND CONCLUSION

The studies presented in this paper have made it possible to define two methods that use relatively unsophisticated and limited equipment. The packing cases used for transporting the equipment are used as work table and shelter during manipulations. The list



Fig. 7. Plantlets in sand 2 weeks after transfer from culture

of equipment required for both methods is given in the Materials and Methods section.

In addition to the calcium hypochlorite and water to prepare the sterilizing solution, storage of solid endosperm cylinders only requires 1 jar per embryo. However, under current conditions, the direct-culture method involves the use of a jar of sterile water, a culture tube containing the medium and a Petri dish for 5 embryos. Simplification may be possible and studies are being conducted on replacing calcium hypochlorite by sodium hypochlorite (eliminating the need to filter the disinfecting product and to carry 10 litres of water).

Direct inoculation into culture in the field necessitates the removal of the actual embryo from the cylinder of endosperm. Although familiarization with the technique is required, it is simple to carry out.

Embryo contamination and development in vitro

The contamination rates observed for the two methods described are low. Culturing in the open air on sterile medium gives a contamination rate of 10%. This is highly satisfactory and the

method can be considered as operational, particularly as embryo development is obtained without any further manipulations. Embryo development is completely analogous to that of embryos cultured in the laboratory, even when the culture tubes are kept under a simple, non-air-conditioned shelter for 2 months.

For short journeys, the storage of solid endosperm cylinders containing the embryo in a KCl solution offers the advantages of speed and simplicity. The contamination rate after the second disinfection and transfer to axenic conditions is very low (5%) and identical to that obtained for immediate inoculation into culture in the laboratory. However, gemmule development is significantly delayed after storage for a period of 14 days in KCl. It should be noted that observations have shown that appearance of the gemmule could be delayed by 10-12 months: if the additional examinations currently being made indicate good rates of plantlet development, storage in KCl could represent a practical short-term preservation technique.

To conclude, the two methods described can be used for coconut germplasm-collecting operations. If the collecting sites are near the *in vitro* culture laboratory, the method of solid endosperm cylinder sampling and storage may be preferable, as it is simple and quick. For storage lasting more than a week, direct inoculation into culture gives better results, and the cultured embryos can be stored for at least 2 months under normal climatic conditions without disrupting the rate of development or increasing the amount of contamination.

There are numerous possibilities for further improving this technique, both in the definition of equipment requirements (reduction in bulk, development of standard instruments) and in the control of embryo development (rate of development of the gemmule and percentage success). Our studies are continuing along these lines, as well as on the transfer of plantlets to natural growing conditions and their subsequent development.

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RESUME

La culture in vitro d'embryons zygotiques résoud les problèmes que posent le transport et le stockage des noix de coco (Cocos nucifera L.), en particulier lorsqu'elles sont collectées à l'occasion de projections sur le terrain. On a mis au point des méthodes d'échantillonnage et de stockage à court terme des embryons. L'ensemencement direct de milieux de culture, permettant la croissance de l'embryon dans des conditions axéniques, a été effectué avec succès sur le terrain. Une autre méthode consiste à placer les embryons dans leur endosperme dans une solution saline pendant de brèves périodes avant prélèvement et repiquage sur cultures en laboratoire. Les techniques et le matériel utilisés sont décrits dans ce document, ainsi que le développement ultérieur des embryons et le transfert des plantes dans des conditions de végétation naturelles.

RESUMEN

El cultivo in vitro de embriones cigóticos resuelve los problemas que plantea el transporte y almacenamiento de semillas de coco (Cocos nucifera L.), especialmente cuando se obtienen en condiciones de recolección normales. Se han preparado métodos de muestreo y conservación a corto plazo de embriones. En las condiciones normales de recolección se ha conseguido efectuar la inoculación directa en medios de cultivo que permiten el crecimiento de los embriones en condiciones axénicas. Otro método consiste en mantener los embriones en núcleos de endosperma en una solución salina durante breves períodos de tiempo antes de realizar la excisión e inoculación en cultivo en un laboratorio. En el presente artículo se describen las técnicas y el equipo utilizados, así como el posterior desarrollo de los embriones y la transferencia de las plantas a condiciones naturales de crecimiento.

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